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Diversity Outbred Mice Identify Population-Based Exposure Thresholds and Genetic Factors that Influence Benzene-Induced Genotoxicity

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Abstract

Background: Inhalation of benzene at levels below the current exposure limit values leads to hematotoxicity in occupationally exposed workers.

Objective: We sought to evaluate Diversity Outbred (DO) mice as a tool for exposure threshold assessment and to identify genetic factors that influence benzene induced genotoxicity.

Methods: We exposed male DO mice to benzene (0, 1, 10, or 100 ppm; 75 mice/exposure group) via inhalation for 28 days (6 hr/day for 5 days/week). The study was repeated using two independent cohorts of 300 animals each. We measured micronuclei frequency in reticulocytes from peripheral blood and bone marrow and applied benchmark concentration modeling to estimate exposure thresholds. We genotyped the mice and performed linkage analysis.

Results: We observed a dose dependent increase in benzene induced chromosomal damage and estimated a benchmark concentration limit of 0.205 ppm benzene using DO mice. This estimate is an order of magnitude below the value estimated using B6C3F1 mice. We identified a locus on Chr 10 (31.87 Mb) that contained a pair of over-expressed sulfotransferases that were inversely correlated with genotoxicity.

Conclusions: The genetically diverse DO mice provided a reproducible response to benzene exposure. The DO mice display inter-individual variation in toxicity response and, as such, may more accurately reflect the range of response that is observed in human populations. Studies using DO mice can localize genetic associations with high precision. The identification of sulfotransferases as candidate genes suggests that DO mice may provide additional insight into benzene induced genotoxicity.

Introduction

Benzene is a ubiquitous airborne pollutant that is classified as a carcinogen (IARC 2012; U.S. EPA 1998; U.S. NTP 2011a). Human exposure to benzene occurs mainly through inhalation in both environmental and occupational settings (Weisel 2010). The U.S. Occupational Safety and Health Administration has set an eight hour, time weighted average exposure limit of 1 ppm. However, workers exposed to benzene at air concentrations below 1 ppm still show evidence of hematotoxicity (Lan et al. 2004; McHale et al. 2012). Occupational exposures as low as 0.3 ppm have been shown to increase the risk of leukemia and myelodysplastic syndrome (Glass et al. 2003; Schnatter et al. 2012). At the molecular level, benzene exposure alters gene expression in peripheral blood cells (McHale et al. 2009), induces aneuploidy in hematopoietic progenitor cells (Zhang et al. 2012) and induces chromosome damage in myeloid progenitor cells (McHale et al. 2008). These and a multitude other effects including AhR dysregulation, reduced immunosurveillance and oxidative stress probably lead to benzene-induced hematologic malignancies (McHale et al. 2012). These and a multitude other effects including AhR dysregulation, reduced immunosurveillance and oxidative stress probably lead to benzene-induced hematologic malignancies (McHale et al. 2012).

Benzene metabolism and clearance varies significantly in the human population (Dorne et al. 2005; Hattis et al. 1987) and these differences may be influenced by both past or current co-exposures as well as genetic factors. Occupational studies of benzene exposure focusing on candidate genes have quantified the effects of polymorphisms on hematotoxicity and genotoxicity end points (Garte et al. 2008; Kim et al. 2007; Lan et al. 2005; Lan et al. 2009; Shen et al. 2006). However, the scope and impact of human genetic variation on toxicity responses remains poorly understood.

Exposure thresholds for suspected toxicants are typically determined using animal models. One of the most common mouse models used in toxicology assessments at the National Toxicology Program (NTP) is the B6C3F1 mouse (King-Herbert and Thayer 2006). These mice are derived by crossing two inbred strains, C57BL/6J and C3H/HeJ, and, like identical twins, all B6C3F1 mice are genetically identical (i.e. isogenic). The use of isogenic mice has been considered an advantage in toxicology studies because it should reduce the within-group variance, which in turn reduces the number of animals required to detect a specified level of response. However isogenic mice fail to model the influence of genetic diversity on toxicity responses, a critical factor in human responses to toxicants. Furthermore, inadvertent selection of a strain with an idiosyncratic response could result in significant bias and compromise the reliability of safe exposure estimates.

There has been increased interest in incorporating genetic variability into human health risk assessments (King-Herbert et al. 2010; Zeise et al. 2013). One approach involves testing toxicants in mice that are genetically diverse rather than isogenic. Genetic diversity can be incorporated by using panels of inbred mice, such as the Mouse Diversity Panel (McClurg et al. 2007), the Collaborative Cross (Threadgill et al. 2011), or by using populations of outbred mice (Yalcin et al. 2010). Outbred mice offer several advantages over inbred mice; like humans, they are genetically heterozygous and carry a complex mixture of alleles. Each animal in an outbred population is genetically unique which leads to more efficient study designs compared to strain panels (Belknap 1998). However, many commercially available outbred stocks suffer from limited genetic diversity (Yalcin et al. 2010) and cryptic population structure (Aldinger et al. 2009).

The Diversity Outbred (DO) mice are a recently developed population derived from eight inbred founder strains (Svenson et al. 2012). The founder strains have been fully sequenced and contribute approximately 38 million SNPs and 7 million structural variants to the DO (Keane et al. 2011; Yalcin et al. 2011) - a level of genetic diversity that is similar to humans (Ideraabdullah et al. 2004). Here we use DO mice to assess a toxicity response to benzene exposure and to estimate a benchmark concentration (BMC) of benzene exposure that is likely to result in a 10% increase in chromosomal damage over baseline. We show, despite significant variation in benzene induced chromosomal damage among DO mice, that benchmark concentration estimates are repeatable across different study cohorts. Furthermore, estimates are lower by an order magnitude compared to estimates derived from previous reports based on an isogenic mouse model. In addition to toxicity screening the unique genetic structure of the DO mice can be used to perform high-resolution linkage mapping to identify genetic variants that determine individual responses to toxicant exposures. This study demonstrates the utility of DO mice for the estimation of exposure thresholds and for discovery of the genetic basis of variation in toxicant responses.

Materials and Methods

Benzene inhalation exposure

We obtained 600 male DO mice (J:DO; Stock Number 009376), between three and five weeks of age, from The Jackson Laboratory (Bar Harbor, ME). The mice were received in two cohorts of 300 mice each from the 7th and 8th generation of outbreeding, respectively, which were delivered four months apart. We provided the mice with NIH-31 diet (U.S. NIH) *ad libitum* and filtered tap water. Mice were singly housed in cages with irradiated, heat treated hardwood bedding (Teklad, Indianapolis, IN). Mice were acclimated for between 11 and 18 days and were

5-8 weeks of age at the start of the exposures. We randomized the mice after weight stratification to each exposure group such that the mean body weight was similar ($\pm 20\%$) in each exposure group. We exposed 75 mice in each cohort to one of four benzene concentrations of 0 (filtered air), 1, 10 or 100 parts per million by volume (ppmv). We exposed the mice in whole body inhalation chambers for 6 hours per day, 5 days per week for a total of 26 to 28 exposures. The mean daily exposure concentrations for cohort 1 were: 1.001 ± 0.003 , 10.07 ± 0.02 , and 100.13 ± 0.11 ppm. For cohort 2, they were 1.000 ± 0.003 , 9.99 ± 0.12 , and 100.18 ± 0.10 ppm. Quality control data for the exposure chambers is available upon request from the corresponding author. We rotated cages within a rack and chamber over time using standard protocols as described in (Office of Laboratory Animal Welfare 2002; U.S. NTP 2011b). We euthanized the mice by CO₂ asphyxiation (95% CO₂ + 5% O₂) on the morning following the last exposure. These studies were conducted under NIH Guidelines and were approved by the NIEHS Institutional Animal Care and Use Committee. Animal care and experimental design followed all applicable NIH protocols and standard operating procedures to alleviate animal suffering or deprivation.

Tissue collection

We collected blood before the first exposure via the tail vein and stored it in a heparin containing Vacutainer® (Becton Dickinson & Co., Franklin Lakes, NJ). At euthanasia, we collected blood via cardiac puncture into EDTA Vacutainer® tubes (Becton Dickinson & Co., Franklin Lakes, NJ). We collected bone marrow by aspirating the right femur with Heat Inactivated Fetal Bovine Serum (HI FBS) for flow cytometry micronucleus analysis (Witt et al. 2008).

Micronucleus assay

We used micronucleus (MN) frequency in reticulocytes (RET) and mature erythrocytes (ERC) as a quantitative measure of chromosomal damage. Micronuclei are chromosomes or chromosomal

fragments that fail to incorporate into either of the two daughter nuclei after karyokinesis. During formation of the reticulocyte in the bone marrow, micronuclei not extruded with the main nucleus are readily visualized and quantified, providing a simple, accurate assessment of chromosomal damage (Heddle 1973; Witt et al. 2008). We isolated RETs and ERCs from peripheral blood (PB) before the first exposure and in PB and bone marrow (BM) at euthanasia. Samples were processed immediately upon collection as described in the MicroFlow® BASIC kits (for mouse) (Litron Laboratories, Rochester, NY) and as described in (Witt et al. 2008). We counted cells and assessed MN frequency in RETs and ERCs using flow cytometry. RETs were identified by the presence of an active transferrin receptor (CD71+) on the cell surface; CD71-negative cells were identified as mature ERCs. We evaluated approximately 20,000 RETs and 10^6 mature ERCs for each animal (Kissling et al., 2007). In addition, the %RET was determined in approximately 10^6 ERCs. MN data were normalized to counts per 1000 cells. We calculated the means and standard deviations on the log scale and transformed them back to the original scale because MN-RETs and MN-ERCs are log-normally distributed. We report the geometric mean of MN-RETs and MN-ERCs along with the antilog of the confidence limits (Bland and Altman 1996). We successfully measured MN-RET in PB for 592 mice and in BM for 570 mice.

Statistical comparisons

We estimated the effect of cohort and exposure concentration using two-way analysis of variance on the \log_2 transformed values. We calculated the mean and standard deviation (s.d.) of MN-RET on a log scale and took the antilog. We report the geometric mean and confidence interval (C.I.) as the geometric mean \pm 2s.d. We used Tukey's honest significant difference to estimate phenotype differences between exposure groups (Hsu 1996).

Benchmark concentration modeling

We used the MN-RET measurements from both cohorts, separately and combined, to estimate exposure thresholds using benchmark concentration (BMC) modeling (Crump 1984). This method is recommended by the U.S. EPA's Guidelines for Carcinogen Risk Assessment (U.S. EPA 2005) and is implemented in the EPA Benchmark Dose software (BMDS, v2.4), which was used for all BMC analyses. We followed the methodology described in the Technical Guidance Document 2012 (U.S. EPA 2012a, b) for BMC modeling.

The benchmark response (BMR) is the minimum increase in MN-RETs above the control group mean that is considered to be biologically significant. BMR is used to define an associated benchmark concentration, which is the concentration of benzene that is expected to result in the specified BMR. We evaluated the BMC using a BMR defined as a 10% increase (BMR_{10}) and one standard deviation (BMR_{1SD}) increase in MN-RET above the 0 ppm group mean. The benchmark concentration limit (BMCL) is the lower bound of a one-sided 95% confidence interval around the BMC.

We fit the U.S. EPA recommended models for continuous data, including Hill, linear, second order polynomial, power and exponential models. The exponential model (Model 4 in BMDS) provided the best fit to the data (goodness of fit $p > 0.1$) and was used to calculate the BMC and BMCL. The exponential continuous data model was:

$$\mu(X) = a[c - (c - 1)e^{-bX}] \quad (1)$$

where $\mu(X)$ is median response at exposure concentration X , a is background response, b is slope and c is an asymptote parameter (U.S. EPA 2012b). We used the BMDS software to fit the model using all exposure groups but found that the model fit was improved by removing the 100

ppm exposure group. As suggested by the E.P.A. guidance, we fit the model using only the 0, 1 and 10 ppm groups. This choice is motivated by the desire to accurately model responses at the lower end of the exposure range.

We obtained exposure-response data for reticulocytes in bone marrow of B6C3F1 mice at 4 weeks from (Farris et al. 1996). B6C3F1 are F1 mice derived by crossing C57BL/6J and C3H/HeJ mice. We calculated the BMCL for the B6C3F1 mice using the same methods as for DO mice.

Summary files for these analyses are included in the Supplemental Material.

Genotyping

We collected a tail biopsy from each mouse and flash froze it in liquid nitrogen. We sent the tail tips to GeneSeek (Lincoln, NE) for DNA isolation and genotyping on the Mouse Universal Genotyping Array (MUGA)(Collaborative Cross Consortium 2012), which contains 7,854 probes distributed across the mouse genome. The mean sample call rate was 94.7% (+/- 1.2%). Seven samples with allele calls rates below 90% were removed from the genotyping data. The genotype data is available in the Supplemental Material at http://churchill.jax.org/publications/French_EHP_2014.shtml.

Linkage mapping

Using the genotype data from the MUGA, we reconstructed the genomes of each DO mouse in terms of the founder haplotypes using a hidden Markov model (HMM)(Gatti et al. 2014). Briefly, at each marker, the HMM estimates the haplotype contribution of each of the eight founders to each mouse. We performed linkage mapping by regressing $\log(\text{MN-RET}/1000 \text{ RETs})$ on the haplotype contributions at each marker. The regression model includes an

adjustment for relatedness among individual DO mice (Cheng et al. 2011). We mapped log(MN-RET) in PB and BM using 144 animals with complete data from the two 100 ppm exposure groups. Linkage analysis provides a likelihood ratio statistic (LOD) at each marker and eight regression coefficients that estimate the additive effects of founder alleles. Genome-wide significance thresholds for linkage mapping were determined by analyzing 1,000 permutations of the phenotype data (Churchill and Doerge 1994). We used the Bayesian credible interval method to define the quantitative trait locus (QTL) peak support interval (Sen and Churchill 2001).

Association mapping

The eight DO founders have been fully sequenced (Keane et al. 2011; Yalcin et al. 2011). We used the haplotype reconstructions of the DO mice to impute the founder sequences onto the DO genomes within the Chr 10 linkage peak interval and mapped log(MN-RET) by additive regression on SNP genotypes (Baud et al. 2013; Gatti et al. 2014).

Heritability

We estimated heritability based on the genetic and environmental variance components for pre-exposure peripheral blood MN-RET. Using the R package *regress* (Clifford and McCullagh, Commonwealth Scientific and Industrial Research Organization, Australia, R Foundation for Statistical Computing, Vienna, Austria), we fit a mixed model in which the covariance structure was a linear combination of the kinship matrix and the identity matrix and estimated the narrow-sense heritability as the ratio of additive genetic variance over total variance.

Test for dominance

We compared three models in order to test for dominance of the CAST/EiJ (CAST) allele at the Chr 10 QTL. We extracted the CAST allele proportion from the haplotype reconstructions at

31.8682 Mb on Chr 10. We coded the additive effect of the CAST allele as 0, 0.5 or 1, depending on whether the CAST allele was missing, heterozygous or homozygous, respectively, in each mouse. We coded the dominant effect of the CAST allele as 0 if the mouse had no CAST allele and 1 if the mouse had at least one copy of the CAST allele. We fit an additive model, a dominant model and a general model with both an additive and dominant effect. We performed a likelihood ratio test comparing the general model with the additive and dominant models separately.

Founder gene expression

We obtained founder gene expression in liver, kidney and spleen for the genes in the Chr 10 QTL interval from the Gene Expression Survey of 26 Inbred Mouse Strains (The Jackson Laboratory 2014). Briefly, expression was measured in the liver, kidney and spleen of male and female ($n = 5$ per sex) inbred mice from 26 inbred strains on the Illumina Sentrix Mouse 6 v1.1 platform. Probes containing Sanger SNPs (see next paragraph) were removed from the analysis. Reported values are \log_2 normalized intensity.

Mouse genes and SNPs

We obtained mouse genes from the Mouse Genome Database, v 5.18 (Blake et al. 2014). We obtained the founder SNPs and structural variants from the Sanger Mouse Genomes Project, REL-1303 (Keane et al. 2011; Yalcin et al. 2011). All genomes coordinates are on Genome Reference Consortium Mouse Genome Build 38 (GRCm38).

Supplemental Material. All data from this study as has been posted at http://do.jax.org/publications/French_EHP_2014.shtml.

Results

We exposed two independent cohorts of 300 male DO mice to benzene at 0, 1, 10 or 100 ppm and measured micronucleated reticulocytes and erythrocytes, a marker of chromosomal damage in red blood cells. We estimated exposure thresholds using benchmark concentration modeling for each cohort separately and for the combined data. We performed linkage mapping to discover genomic loci that are associated with benzene induced chromosomal damage in reticulocytes using DO mice in the combined 100 ppm exposure groups.

Benzene induced chromosomal-damage

Before exposure, we measured MN-RET and MN-ERC in peripheral blood and found no difference between exposure groups (2-way ANOVA $p = 0.56$) or cohort (2-way ANOVA $p = 0.44$) (Figure 1A, Supplemental Material, Table S1). Data are presented only for peripheral blood MN-RET because the data are similar to blood MN-ERC values and the same quantitative trait locus is identified from both data sets (data not shown). The mean pre-exposure MN-RET was 2.17 (1.05, 4.47) MN-RET per 1000 RETs (MN-RET/1000). The estimated heritability of pre-exposure MN-RETs in PB was 0.65, which indicates that the genetic variance is roughly two times larger than the environmental variance.

After four weeks of exposure to benzene, we measured MN-RET in peripheral blood and found a significant difference between exposure groups (2-way ANOVA $p < 10^{-16}$). No significant differences were observed within dose groups across the two cohorts (Figure 1B; Supplemental Material, Table S2). The mean in the 0 ppm group was 2.68 MN-RET/1000 (1.33 – 5.39). There was no difference in MN-RET means between the 0 ppm and 1 or 10 ppm exposure groups (0 & 1 ppm Tukey HSD p -value = 0.93; 0 & 10 ppm $p = 0.074$). The mean in the 100 ppm exposure

group increased to 14.6 MN-RET/1000 (2.29 – 92.7, $p = 5.03 \times 10^{-10}$), corresponding to a 573 percent increase from baseline.

The Pearson correlation between pre- and post-exposure PB MN-RET were 0.60, 0.63, 0.54, and 0.38 for the 0, 1, 10 and 100 ppm groups, respectively. These values are consistent with the heritability estimate of 0.65 and again indicate that the error variance is roughly half of the genetic variance. Heritability of post-exposure PB MN-RET was estimated to be 0.21.

At the end of the exposure period, we measured MN-RET in bone marrow and observed an increase with increasing exposure concentration (linear model in which bone marrow MN-RET was regressed on exposure concentration, $p < 10^{-16}$) (Supplemental Material, Table S3, Figure 1C). Again there was no significant difference between cohorts (Supplemental Material, Table S3). For the 1 and 10 ppm exposure groups, MN-RET/1000 increase by roughly 1 unit with each order of magnitude increase in benzene concentration. In the 100 ppm exposure group, MN-RET/1000 increased by 9 units above the 10 ppm group. Heritability of post-exposure BM MN-RET was 0.035.

Benchmark concentration modeling

We used the BM MN-RET data to estimate benzene exposure thresholds using the benchmark concentration modeling approach (Crump 1984). This approach involves fitting a model to the exposure/response data, selecting a biologically relevant toxicity response (i.e. BMR), matching this response to an exposure concentration (i.e. BMC) and estimating the lower bound on the confidence interval (i.e. BMCL) as described in Methods.

We fit an exponential model to BM MN-RET using 570 mice in the combined data from all four exposure groups and estimated a $BMCL_{1SD}$ of 13.3 (Table 1 Row 15, Figure 2A) and a $BMCL_{10}$

of 1.52 ppm (Table 1 Row 7, Figure 2B). However, we observed a significant lack-of-fit statistic ($p_{\text{lack-of-fit}} = 0.021$). E.P.A guidelines indicate that it is acceptable to remove the highest concentration in order to improve the model fit at the lower concentrations where the BMCL will be determined (U.S. EPA 2012a). The reasoning behind this recommendation is that lack of fit may be indicative of different type of physiological response at higher exposures. We removed the 100 ppm group measurements and, using data from the 424 mice in the 0 through 10 ppm exposure groups, fit an exponential model to the data. We estimated a BMCL_{10} of 0.205 ppm (Table 1 Row 3, Figure 2C). However, we were unable to calculate the $\text{BMCL}_{1\text{SD}}$ in the 0 through 10 ppm groups because the mean MN-RET plus one standard deviation of the 0 ppm group was greater than the 10 ppm group MN-RET mean. We repeated the benchmark dose analysis separately on each cohort of DO mice and obtained results consistent with the combined analysis (Table 1) to demonstrate reproducibility.

We obtained bone marrow erythrocyte toxicity data from a previously published benzene inhalation study using the same study design in male B6C3F1 mice (Farris et al. 1996) and performed BMC modeling. Using the micronucleated polychromatic erythrocyte (MN-RET) data from bone marrow at 4 weeks, we estimated a $\text{BMCL}_{1\text{SD}}$ of 3.12 (Table 1, Row 16, Figure 2D) and a BMCL_{10} of 3.66 ppm (Table 1, Row 8, Figure 2E). We note that both of these values are an order of magnitude higher than the BMCL_{10} estimated using DO mice. We were unable to estimate a BMCL_{10} using the 0, 1 and 10 ppm exposure groups because the control group mean MN-PCE plus 10% was greater than the 10 ppm group MN-RET mean.

Linkage mapping analysis

We genotyped each mouse at 7,854 markers and performed linkage mapping on post-exposure MN-RET in blood and bone marrow using 144 animals with complete data in the combined 100 ppm exposure groups.

We identified a single significant locus on Chr 10 shared by both PB and BM MN-RET (Figure 3A, Supplemental Material, Figure S1). We describe BM MN-RET findings here and provide results of PB analysis in the Supplemental Material (Supplemental Material, Tables S1 & S2, Figure S1). The maximum LOD score (20.7, $p < 0.01$) occurred at 31.87 Mb on Chr 10 (Figure 3B) and accounted for 48.7 % of the phenotypic variance. We estimated the additive effect of each founder allele and observed that CAST/EiJ alleles are associated with lower levels of MN-RET (Figure 3B). Comparing the goodness-of-fit for genetic models of additive ($p = 2.4 \times 10^{-7}$) and dominant ($p = 0.41$) effects, we conclude that the CAST allele acts dominantly (Figure 3C). Mice carrying at least one copy of the CAST allele on Chr 10 are more resistant to benzene induced chromosomal damage. We performed a second linkage mapping analysis with the Chr 10 QTL regressed out but did not find any additional QTL at the genome wide $p < 0.05$ level.

The support interval for the QTL on Chr 10 spans from 31.8 to 34.2 Mb and contains 21 annotated genes. In order to narrow this list of candidate genes, we carried out association mapping on imputed SNPs, looked for functional SNPs and small insertion-deletion polymorphisms in coding regions, examined external data resources to look for expression differences, and examined genomic sequence reads to look for large-scale structural polymorphisms. Several lines of evidence point to two of these genes, *Gm4794* (Gene model 4794) and *Sult3a1* (Sulfotransferase 3A1), as strong candidate genes for increased resistance to benzene-induced chromosomal damage.

We imputed the sequences of the individual DO genomes (Gatti et al. 2014) and performed association mapping at each SNP within the Chr 10 interval (Figure 3D). While this approach can be helpful in narrowing the candidate gene list, in this case CAST contributes unique alleles that cover the entire QTL interval and these SNPs all have strong association with MN-RET. We found CAST-specific SNPs and small insertion-deletion polymorphisms in transcribed exons of 9 of the 21 candidate genes (Keane et al. 2011; Yalcin et al. 2011): *Clvs2*, *Dse*, *Gm15939*, *Gm4794*, *Rsph4a*, *Rwdd1*, *Sult3a1*, *Trdn*, and *Zufsp*.

We looked for CAST specific expression differences in the DO founder strains and in an independent population of untreated DO mice (Munger et al. 2014). Three genes had differential expression in CAST; *Hddc2*, *Gm4794* and *Sult3a1*. *Hddc2* (HD domain containing 2) had higher expression in liver, kidney and spleen. However, in the liver expression QTL (eQTL) data, it had a local eQTL in which both the CAST and PWK alleles were associated with higher expression. This is not consistent with the MN-RET allele effects (Figure 3B). *Gm4794* and *Sult3a1* showed higher constitutive liver expression of CAST mice among the founder strains (Supplemental Material, Figure S2), but are not expressed in kidney or spleen (data not shown). *Gm4794* and *Sult3a1* also have a strong liver eQTL for which the CAST allele increases expression (Figure 4). These eQTL are local and *cis*-acting.

We examined the genomic sequencing data of the founder strains in the QTL interval and found that CAST mice have a 350 Kb segmental duplication (33.52 to 33.87 Mb) that contains 4 genes: *Clvs2*, *Gm15939*, *Gm4794*, and *Sult3a1* (Supplemental Material, Figure S3). *Clvs2* is expressed in neurons and does not appear to be expressed in liver or bone marrow (Wu et al. 2009). *Gm15939* is a predicted gene that is thought to be not transcribed. *Gm4794* is a paralog of *Sult3a1* that is transcribed and produces a protein product (ENSMUSP00000127159.1). It

contains a sulfotransferase domain and shares 84% amino acid sequence identity with *Sult3a1*. There are no recombination events in our DO mice in the duplication region that could help to distinguish the effects of *Gm4794* from *Sult3a1*.

Discussion

Implications for benchmark concentration modeling

The current U.S. EPA IRIS risk assessment benzene BMCL of 7.2 ppm is based upon lymphocyte count data from 44 human subjects in an occupational exposure assessment (Rothman et al. 1996). In 2004, the same research consortium observed that workers exposed to less than 1 ppm benzene had reduced white blood cell counts and circulating hematopoietic stem cells (Lan et al. 2004), indicating that additional research is necessary to determine appropriate risk thresholds for benzene exposure.

We performed BMC modeling on inhalation exposure data from DO mice and also on data from a previous study of B6C3F1 mice (Farris et al. 1996). Our estimated BMCL₁₀ using DO mice, at 0.205 ppm, was an order of magnitude lower compared to values obtained using B6C3F1 mice. The DO-based estimate of BMCL₁₀ is consistent with observed exposure toxicity in human subjects (Lan et al. 2004) and thus may provide a more appropriate starting point for establishing exposure thresholds that protect the most sensitive human subpopulations.

There are several aspects in which our study may have improved inhalation exposure assessment for benzene. We examined exposure concentrations of 0, 1, 10, and 100 ppm. In retrospect it would have been preferable to add an exposure between 10 and 100 ppm to evaluate the fit of the standard dose-response model. In addition, because the estimated BMCL₁₀ values fall below 1 ppm, an exposure group at a concentration between 0 and 1 ppm would increase our confidence

in this estimate. However, low concentration exposures below 0.5 ppm are difficult to control in standard flow chambers. There may also be variation in respiration rates between DO mice and measuring the respiration rates may improve our ability to characterize the sources of variation in our study.

In the analysis of our data we followed U.S. EPA recommended guidelines for BMC modeling but we encountered a number of problems. The significant lack of fit when the high dose group was included suggested that the recommended models do not accurately reflect responses over the full range of exposures studied. Removing the high exposure group data improved the overall model fit, but it would have been preferable to evaluate all of the data. In addition, when applying the recommended method for computing BMC, both our data and the B6C3F1 data failed to meet expectations implicit in the procedure. Extrapolation of risk estimates to low exposures is a challenging problem and well-defined procedures are required to assure that consistent regulatory recommendations are provided. However, our experience in applying these procedures suggests that more research may be needed to identify new methods that conform to the realized properties of exposure-response data.

Advantages of DO mice in toxicology

The use of isogenic mice, like B6C3F1, has been common practice for over 30 years (King-Herbert and Thayer 2006). Isogenic lines are assumed to produce lower variance and thus require smaller sample sizes compared to genetically diverse mice. This appears to be an advantage. However, isogenic mice do not reflect the variation in toxicity responses that we expect in a genetically diverse population like humans. Furthermore, inadvertent selection of a strain (or F1 hybrid) model with an idiosyncratic response could result in significant bias and compromise the reliability of BMC estimates. These results may challenge current paradigms in toxicology

testing, but these data suggest implicit benefits in modeling genetically diverse populations of test organisms.

The observed variation in MN-RETs in our study of DO mice was similar to isogenic B6C3F1 mice in the no-exposure group and ranged up to 9 times greater (3x on SD scale) in the 100ppm exposure conditions (Supplemental Material, Table S3). There are difficulties in directly comparing studies that were carried out at different times and locations. However, we can obtain direct estimates of the genetic contribution to variance from the DO study. Heritability estimates suggest that sample sizes of DO mice required to obtain the same precision as with isogenic mice would need to be increased by a factor of three. The increased cost is offset by the potential to obtain more realistic and generalizable estimates of toxicity response in the genetically diverse populations. While it seems premature to make a general recommendation regarding sample sizes for DO-based toxicology studies, 75 animals per group is adequate to obtain reproducible estimates of BMC and BMCL.

DO mice encompass much greater genetic diversity than the classical inbred strains and commercial outbred stocks. Randomized breeding and genetic monitoring are employed to ensure constant population allele frequencies over time. In addition, the Collaborative Cross strains provide a resource of reproducible inbred animals from the same genetic background as the DO (Collaborative Cross Consortium 2012). This genetic reference population could be useful for validation and mechanistic studies of results obtained in DO mice.

In this study the presence of CAST alleles on Chr 10 resulted in a significantly reduced response to benzene exposure. If, hypothetically, a mouse strain related to CAST had dominated mouse research in the same way that the C57BL/6 strain has, our assessment of exposure risk might

look quite different. This illustrates the arbitrary nature of using a single inbred strain for exposure modeling. No single animal model can provide a perfect surrogate for evaluating toxicity in humans. However, the genetically diverse DO model provides a population-based assessment with levels of interindividual variation that more accurately reflect human populations.

Identification of QTL associated with susceptibility to benzene exposure.

Using 144 DO mice in the combined 100 ppm exposure groups, we identified a region on Chr 10 in which mice carrying at least one CAST allele showed decreased chromosomal damage after exposure. Two genes, *Sult3a1* and *Gm4794*, showed increased hepatic expression in CAST/EiJ when compared to other founder strains. We hypothesize that a genomic duplication specific to the CAST strain leads to increased expression of *Sult3a1* and *Gm4794* in the liver. These genes could play a protective role by sulfating benzene metabolites, such as phenol, before they can be transported to the bone marrow. In humans, sulfate conjugation of benzene metabolites such as phenol is thought to occur in the liver (McHale et al. 2012). Although humans lack a direct ortholog of *Sult3a1*, the human sulfotransferase with the closest amino acid similarity is a phenol sulfotransferase called SULT1A1 (Brix et al. 1999; Gamage et al. 2006). Humans contain between one and five copies of SULT1A1 (Gaedigk et al. 2012; Hebbring et al. 2007; Yu et al. 2013) and our results suggest that copy number variation could be associated with the variation in benzene induced toxicity in humans.

We did not find any significant association near genes that are traditionally associated with benzene metabolism – such as *Cyp2e1*, *Ephx1*, *Sult1a1*, *Mpo* and *Nqo1* - in the 100 ppm exposure group. This may be due to a lack of functional polymorphisms in these genes in the

DO, to the relative importance of these genes to benzene metabolism and clearance in the 100 ppm exposure group, or to differences in benzene metabolism between mice and humans.

Conclusions

We have demonstrated that DO mice experience a reproducible dose-dependent increase in bone marrow MN-RET after benzene exposure by inhalation. We carried out a standard benchmark concentration modeling analysis using the DO mice and found BMCL₁₀ estimates were lower by an order of magnitude compared to an isogenic mouse model. We used genetic mapping analysis of DO mice to identify a locus on Chr 10 that is associated with benzene-induced genotoxicity. Analysis of gene expression and genomic sequencing data provide evidence in support of sulfotransferases as candidate genes underlying the QTL effect.

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Table 1. BMCL for benzene induced MN-RET in DO and B6C3F1 mice.

Row	Strain/population	Doses	AIC	BMR	BMCL*
1	DO: Cohort 1	0 – 10	-81.2	10%	0.148
2	DO: Cohort 2	0 – 10	-88.3	10%	0.167
3	DO: All	0 – 10	-176	10%	0.205
4	B6C3F1	0 – 10	7.56	10%	NA
5	DO: Cohort 1	0 – 100	-47.2	10%	1.25
6	DO: Cohort 2	0 – 100	6.76	10%	1.46
7	DO: All	0 – 100	-37.2	10%	1.52
8	B6C3F1	0 – 100	47.8	10%	3.66
9	DO: Cohort 1	0 – 10	-81.2	1 SD	NA
10	DO: Cohort 2	0 – 10	-88.3	1 SD	NA
11	DO: All	0 – 10	-176	1 SD	NA
12	B6C3F1	0 – 10	7.56	1 SD	NA
13	DO: Cohort 1	0 – 100	-47.2	1 SD	9.87
14	DO: Cohort 2	0 – 100	6.76	1 SD	14.1
15	DO: All	0 – 100	-37.2	1 SD	13.3
16	B6C3F1	0 – 100	47.8	1 SD	3.12

*NA indicates that the BMCL could not be calculated.

Figure Legends

Figure 1. MN-RET measurements are consistent between cohorts. (A) Pre-exposure blood MN-RETs. X-axis shows the benzene dose in ppm and the cohort. Y-axis plots MN-RET/1000 RETs. Boxes plot the median and inter-quartile range. Whiskers cover the entire data range. (B) Post-exposure blood MN-RETs increase in the 100 ppm group. (C) Post-exposure bone marrow MN-RETs increase in a dose dependent manner.

Figure 2. BMC modeling of bone marrow MN-RET in DO mice using the 0, 1, 10, and 100 ppm exposure groups to estimate a BMR_{1SD} (A) and a BMR_{10} (B). Curved black line is the model fit. Points are the mean of each exposure group and bars show the standard error. Dashed blue line is BMC and the red dot and dash line is BMCL. Inset shows detail of low concentration range. (C) BMC modeling of bone marrow MN-RET in DO mice using the 0, 1 and 10 ppm exposure groups using a BMR_{10} . BMC modeling of bone marrow PCE in B6C3F1 mice using a BMR_{1SD} (D) and a BMR_{10} (E).

Figure 3. Linkage mapping of bone marrow MN-RET in the 100 ppm exposure group reveals a significant QTL on Chr10. (A) Solid black line plots the LOD at each marker. Solid horizontal red line is permutation derived significance threshold of $p = 0.05$. (B) Upper panel plots the effects of each of the eight DO founder alleles on Chr 10. The CAST allele (green) is associated with lower MN-RETs. Lower panel plots the LOD score on Chr 10. (C) MN-RET values by DO genotype at the marker with the maximum LOD score on Chr 10 (31.868 Mb). Dots indicate BM MN-RET values for individual DO mice, solid red lines show the mean \pm standard error of each genotype group. Genotypes are listed on the X-axis, with each DO founder represented by a letter. Genotypes containing the CAST allele are shown in green boldface text. (D) Association mapping within the Chr 10 QTL interval. Each dot shows the LOD score at one SNP. Dots in red are above the $p < 0.01$ threshold. Lower panel shows the genes in the QTL interval. Dashed vertical lines show the QTL support interval. *Sult3a1* and *GM4794* are colored red and surrounded by a red box to highlight their location.

Figure 4. Linkage mapping of liver *Sult3a1* and *Gm4794* expression reveals a QTL on Chr 10 in the same location as the MN-RET QTL. (A & B) Black line plots the LOD at each marker. Solid horizontal red line is permutation derived significance threshold of $p = 0.05$. (C & D) Upper

panel plots the effects of each of the eight DO founder alleles on Chr 10. The CAST allele (green) is associated with higher liver *Sult3a1* and *Gm4794* expression. Lower panel plots the LOD score on Chr 10.

Figure 1.

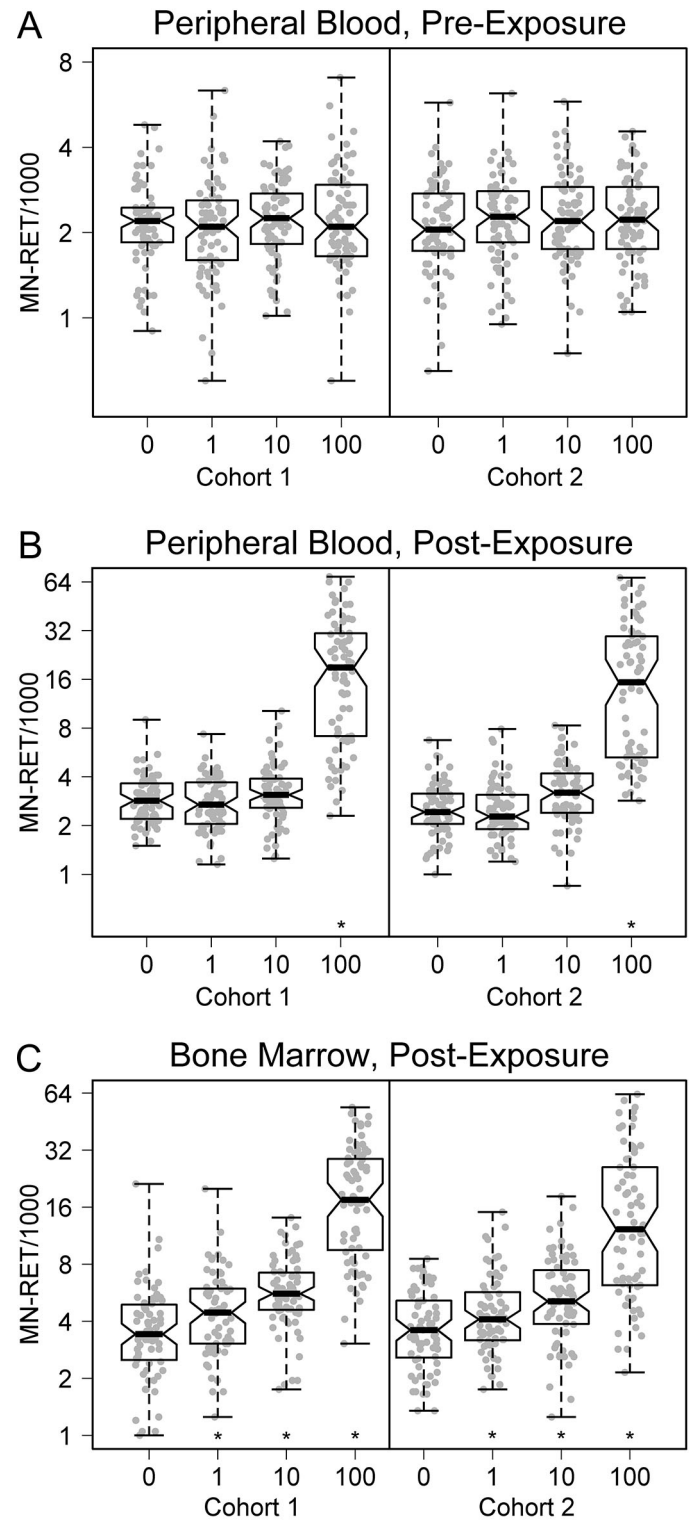
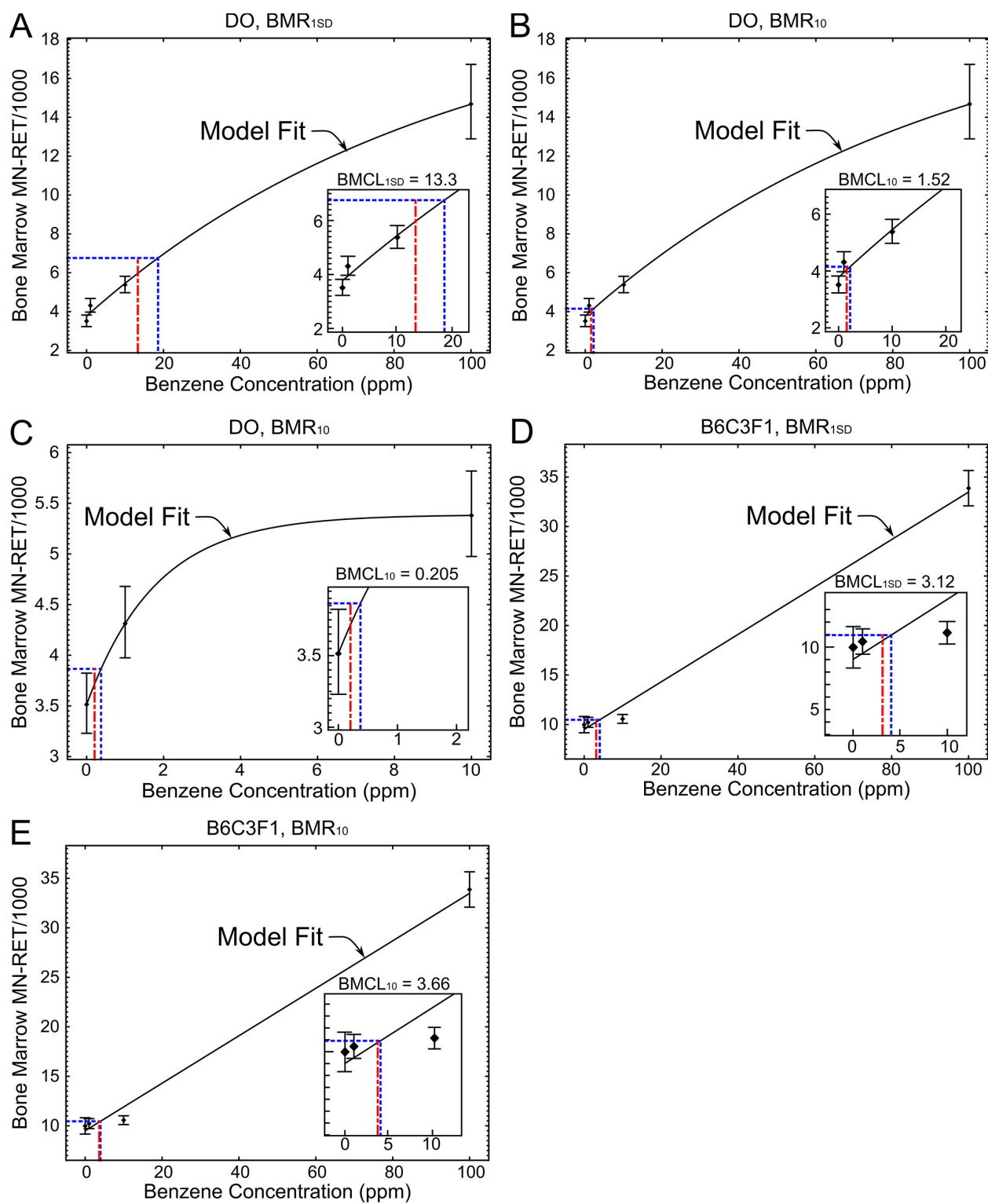


Figure 2.



[illegible]

Figure 4.

